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INTRODUCTION:

The nuclear hormone receptor (NR) superfamily consists of 48 distinct transcription factors in humans. These proteins generally consist of inter-connected functional domains. The N-terminal A/B domain is poorly conserved in size and sequence. The DNA-binding domain (DBD) resides in the center of the polypeptide and binds to DNA response elements upstream of target genes. A hinge region connects the DBD to the ligand binding domain (LBD). The LBD is responsible for binding to the receptor ligand and to some coregulator proteins. There are no three-dimensional structures available for any intact nuclear receptors, or of an A/B domain or hinge region. By contrast, the isolated DBDs and LBDs have been studies by X-ray crystallography in many cases. In the case of the estrogen receptor (ER)-alpha, crystals structures are available for the LBD and DBD as single domains. Since structures consisting of intact or nearly intact polypeptides are entirely absent for this protein family, there is no conceptual framework to help understand their domain-domain interactions. We have therefore undertaken studies to crystallize full-length nuclear receptors including the ER-alpha, and at the same time of PPAR-gamma/RXR-alpha, in several different protein and DNA complexes. Our recent structure determination of the PPAR-gamma/ RXR-alpha heterodimer on DNA have revealed important new biological insights for the nuclear receptor family as a whole, including the estrogen receptor sub-family. We are currently employing several approaches are to help produce a similar crystal structure for ER.

BODY:

Our current efforts in expression and purification of the human ER-alpha protein: We have expressed and purified the full-length hER-alpha protein using a baculovirus and SF-9 expression system. The main protein that comes out of the purification process is, however, smaller than the expected product and has an apparent molecular mass of ~55 kDa. Careful analysis has shown us that this truncation product of ER has lost residues 1-110, but is otherwise complete. This suggests that the 110-C fragment is likely to be relatively well ordered, while the first 110 residues are intrinsically disordered.

Our parallel efforts on the PPAR-gamma protein also demonstrated that most of it's a/B region in the N-terminus is disordered. We were able to prove this definitively when we crystallized the full-length PPAR-gamma with RXR-alpha on DNA. After solving the structure, we could see continuous electron density for all of PPAR and RXR, but neither of their A/B regions. These findings are consistent with our hypothesis that the first 110 residues of ER, an d by extension, the N-terminal regions of many other nuclear receptors are likely to be very poorly ordered.

To make larger quantities of our ER 110-C protein, we have been more recently relying on a new expression system in which we cloned residues 110 through the C-terminus of ER-alpha (designated as ER 110-C) into the pET-46 vector. This systems allows us to more efficiently express the resulting protein in *E.coli* BL21 (DE3) cells. The His-tagged fusion protein is purified using Ni-NTA, Phenyl-Sepharose and gel-filtration chromatography, all in the presence of ER ligand. High affinity ligands such as tamoxifen and estrodial stabilize the protein and allow successful purification, whereas the absence of ligand causes the protein to precipitate in the purification. In the presence of ligands and fairly large bacterial cultures (multi-liter), we obtain enough protein for small scale crystallization trials . The 110-C fragment has been used to generate some crystals, which are unfortunately small, labile (prone to melting and re-appearance). In many cases, the removal of the cover slip from the well leads to immediate crystal dissolution. These characteristics do made the recovery of crystals from the crystallization drop challenging. A secondary difficulty is seen when crystals are harvested and tested, the results suggest a hypersensitivity to radiation damage when testing for diffraction. The reason for this radiation sensitivity may well be due to the unusually high number of cysteine residues in ER, many of which are clustered in the DBD and which could be oxidizing. An alternative explanation is that there is high production of free-radicals in the radiation beam that damage the protein crystals.

Due to the issues discussed above, we are also attempting to implement alternative strategies and approaches in order to generate crystals more readily. To reduce the radiation damage, we are including very high concentrations of the reducing agent DTT in the cryo-soaks. To improve the crystal growth parameters, further fine screenings with gradual increments of solution conditions are being undertaken, in which the concentrations of chemicals, pH, additive solutions, growth temperature and every other parameters are systematically varied to find an optimum condition. The logic is that larger, more dense crystals may be able to better withstand the radiation damage of the X-ray beam. Finally, we are attempting to use micro-crystal seeding to nucleate the production of new crystals, although as yet this has not proven successful.

Alternative approaches being currently pursued relate to the use of low-humidity conditions, which others have found to be a useful means for improving diffraction properties. By equilibrating the crystals against saturated salt solutions inside a glass capillary, the small

amount of mother liquor around the crystal is evaporated away, and the shrinking of the crystal can reduce the volume of the solvent molecules in the lattice. Others have reported a substantial improvement in diffraction when using this technique, and we are currently assessing its success using the ER 110-C protein. Similarly, the usage of small cross-linking molecules (gluteraldehyde) will be tested to see if it can stabilize the crystal lattice to improve both the durability and diffraction of crystals.

In terms of the complex of ER-alpha as a homodimer on DNA, we have now produced a series of differently sized, highly purified duplexes using synthetic strands. All of these duplexes consist of a central bipartite ER response element (with palindromic copies of AGGTCA, separated by the required base-pairs). These duplexes only vary in terms of their overall length and the type and size of their 5' and 3' base overhangs. In the coming months, we will be screening each of these duplexes separately with ER protein homodimers for crystallization.

Very recently, we have been successful in crystallizing and solving the crystal structure of the PPAR-gamma/RXR-alpha heterodimer of full-length nuclear receptors on DNA (Figure 1). Many of the strategies that made this project successful are being adapted at the moment for the ER crystallization project. These include the requirement of DNA duplexes that stack end-to-end in the lattice, the usage of very focused screens with high probability of producing crystals for protein-DNA complexes (these screens are almost entirely based on PEG as the precipitant), and the simultaneous usage of hydrogen-deuterium exchange mass-spectrometry on these protein-DNA complexes to reveal exactly which portions of the proteins are highly exchangeable/mobile, and which parts are stable (Figure 2). The selective removal of the portions undergoing rapid H-D exchange has helped us get the PPAR-RXR/DNA crystals rather quickly. Our recent structure of the PPAR complex is already showing very important new insights about domain-domain interactions that could well be features of the ER polypeptide, including a previously unknown interface between the DBD of one subunit and the LBD of the second (homo- or heterodimeric) polypeptide.

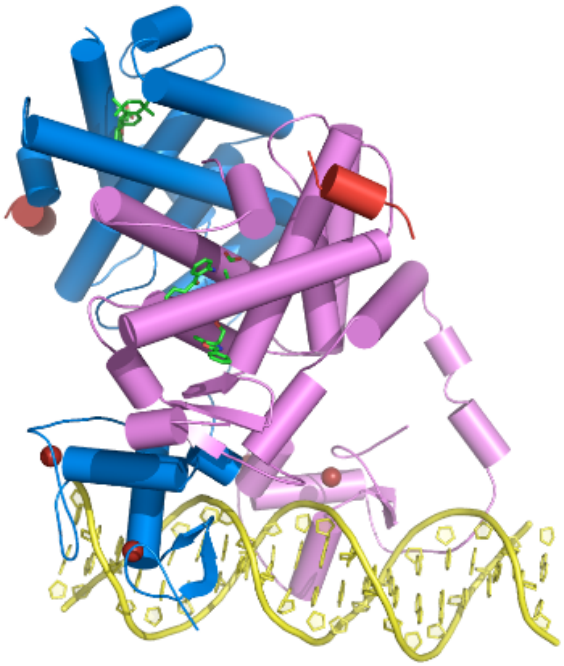
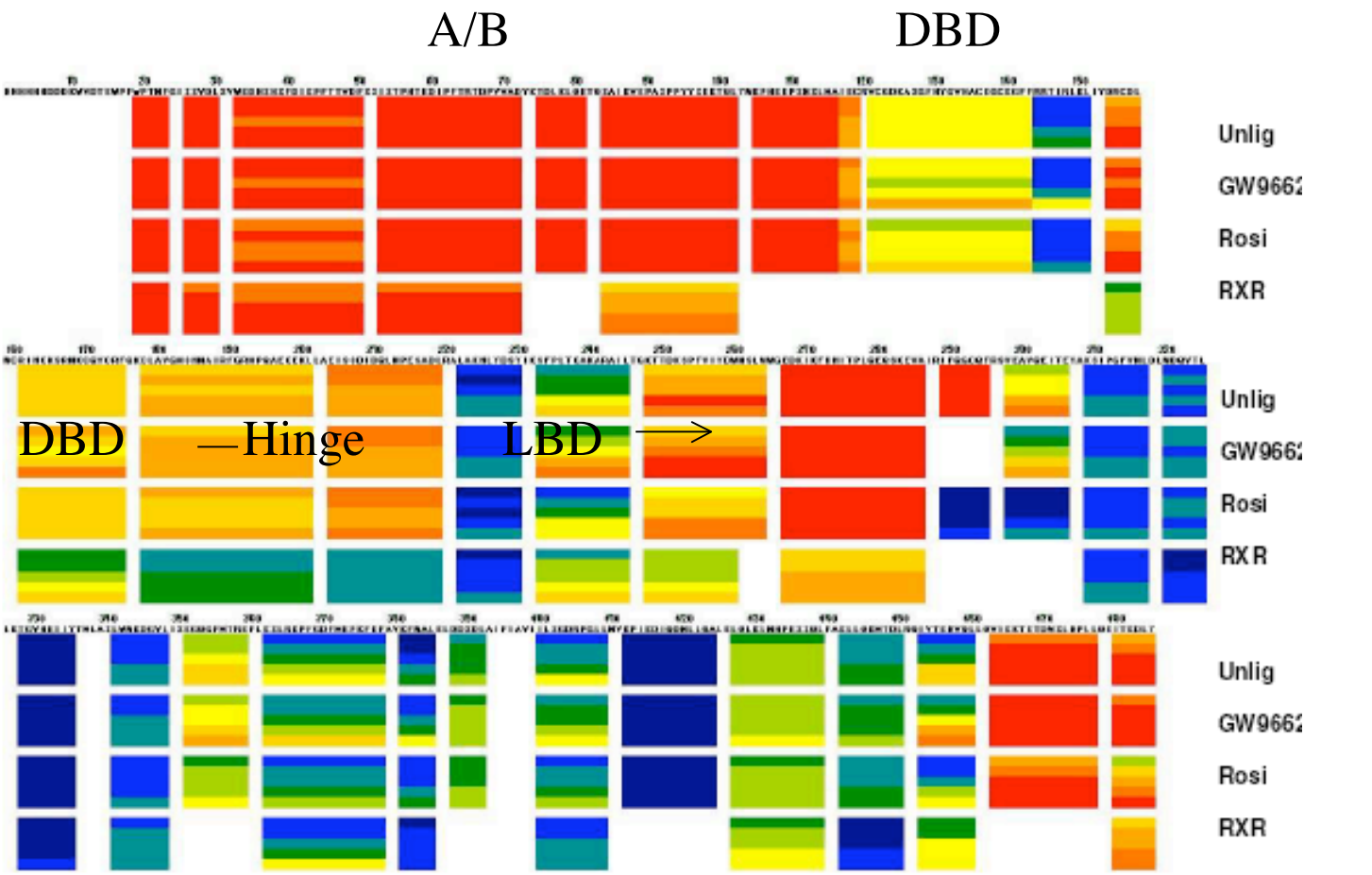


Figure 1 (above): Crystal structure at 3.1 Angstrom of intact PPAR-gamma/RXR-alpha heterodimer bound to PPRE DNA. PPAR is shown in purple, RXR in blue. Red helices correspond to coactivator peptides, and spheres correspond to Zinc ions in the receptor DBDs. Multiple domain-domain interactions are seen for the first time, including those within each polypeptide and from one subunit to another.

Figure 2 (below) : H-D exchange mass-spectrometry on full-length nuclear receptors. The locations of the A/B, DBD, hinge and LBD regions are indicated. Each block represents a peptide region analyzed. Each block contains five time points, 15, 50, 150, 1500 seconds shown in horizontal strips from top to bottom. Empty blocks indicate regions where peptides could not be identified for analysis. "Unlig" shows the data for apo-PPAR, GW9662 and Rosi indicate the presence of each of these ligands, and "RXR" indicates the addition of full-length RXR and PPRE DNA together (and rosiglitazone and 9-cis RA). The deuteration level at each time point is color coded as shown below. This type of data shows that the A/B region in the N-termianl portion of this receptor is highly disordered as it undergoes rapid exchange. Similar data for ER-alpha is now being pursued to better define the boundaries that may be removed to allow for more robust crystal grown.



ACCOMPLISHMENTS:

a) Production of multi-milligram amounts of ER-alpha proteins and duplex DNA
b) Generation of preliminary crystals of ER 110-C protein.
c) Successful crystal structure determination of PPAR-gamma/RXR-alpha full-length proteins on DNA (not yet published).

REPORTABLE OUTCOMES:

Successful RO1 application, with funding from the NIH based on preliminary data related to PPAR-gamma/RXR-alpha heterodimer on DNA.

CONCLUSIONS:

The successful crystallization of a nuclear receptor protein consisting of more than just one domain has been a daunting task over the past 15 years, without a single reported success to date. At the same time, there is strong evidence suggesting that different domains in nuclear receptors are both physically and functionally "wired" together, to mediate their complex responses to ligands, including drugs used in breast cancer therapy – all of which require us to visualize a multi-domain nuclear receptor to fully comprehend. Using the crystallographic expertise of our group, we have in the past few months solved the first crystal structure of intact nuclear receptors PPAR-gamma and RXR-alpha as a heterodimer on DNA, which we are currently writing for publication. At the same time, the goal of achieving a similar success with the ER-alpha protein is still unmet and being fully pursued here. The ER-alpha project will benefit from many of the recent strategies developed here that led to the success with PPAR/RXR. These strategies include approaches in which both intact and A/B truncated polypeptides of ER-alpha are used for crystal growth, DNA duplexes of various sizes are implemented in screens, and various crystal growth and handling improvement techniques are searched that are likely to promote better crystallization and diffraction success. Moreover, we are planning to adapt novel approaches, particularly the use of H-D exchange mass-spectrometry, in order to more conclusively guide us to the most stable regions of ER-protein to be used for future crystallization screens. The goal is to generate the highest quality crystals that can generate the best diffraction data.

MANUSCRIPTS/ABSTRACTS.

None.